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Anti-myc Dithiophosphate DNA Oligonucleotides Selectively Stop the Growth of HL-60 Cells: Synthesis, Purification, and Cell Studies

By

Brandi Ristine Whitley

Biochemistry and Molecular Biology
Sweet Briar College

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This senior honors thesis has been awarded Highest Honors.

Robin Lee Davies
Dr. Robin L. Davies
Sweet Briar College

Jill N. Granger
Dr. Jill N. Granger
Sweet Briar College

Allison B. Jablonski
Dr. Allison B. Jablonski
Lynchburg College

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by Brandi Ristine Whitley

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Research Advisor:
Dr. Robin L. Davies, Sweet Briar College
Second Reader:
Dr. Jill N. Granger, Sweet Briar College
Third Reader:
Dr. Allison Jublonski, Lynchburg College

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ABSTRACT

Whitley, Brandi Ristine. B.S., Sweet Briar College, May 1999. Anti-*myc* dithiophosphate DNA oligonucleotides selectively stop the growth of HL-60 cells: synthesis, purification, and cell studies. Research advisor: Robin L. Davies, Ph.D.

Antisense theory proposes that oligonucleotides (oligo) recognize specific mRNA or DNA sequences and bind to them, thus preventing translation or transcription of a gene. Through the binding of an oligonucleotide to an mRNA that translates an essential protein for cancer growth, the action of the protein is terminated. The product, an oncoprotein, is never formed. Antisense mechanisms are relevant for treating diseases where uncontrolled growth of cells is caused by the overexpression of a gene; cancer is an example. The main difficulty arising from the use of antisense oligonucleotides is that they must be modified in some manner to retain biological activity and non-toxicity but not be recognized by cellular nucleases. Previously, oligos have been modified in several different ways to provide such resistance to degradation and to show affinity for and recognition of the targeted mRNA. This research focuses on a specific type of modification to the DNA oligo backbone: replacement of non-bridging oxygens with sulfur. The monothiophosphate modification (POS) has been studied and shown to have a specific negative effect on cell growth. Dithiophosphate oligos (PS2) are a newer modification in which both non-bridging oxygens are replaced by sulfur atoms at select points. These oligos were synthesized and then purified using reverse-phase high performance liquid chromatography. The targeted oncogene in this research is *myc*; because it is known to overexpress *myc*, the cell line studied was the human leukemia cell line, HL-60. The PS2 oligos showed a decrease in cell growth up to 50%. The POS oligo showed up to a 25% decrease in cell growth as well. These effects were concluded to be sequence specific because the scrambled controls (same base composition as anti-*myc*, but in a random order) for both POS and PS2 did not effect cell growth. The sulfur modification was shown to be non-toxic to the cells as indicated by the normal growth of the cells with scrambled controls. Further research with oligo preparation procedures must be explored to attain more sufficient yields, but the sequence specific mechanism that seems to be occurring in this research will be useful in treatment considerations for different cancers. Different oncogenes should be targeted to determine if the antisense theory is upheld.

Introduction

Cancer has become such a pervasive disease that the interest in studying its pathology and possible cures has heightened. It is estimated that cancer affects three out of four families in the United States alone (35). The disease and its treatments cause substantial mortality and morbidity, prompting intense interest in preventing these obstacles. Most available treatments for cancers are non-specific, meaning that they target all rapidly growing cells, both normal and cancerous. Consequences of these treatments include side effects towards the normal cells. In addition, cancer is a genetically unstable disease. Cancer cells can develop drug resistance through repeated rounds of mutation and selection. This may render a particular non-specific chemotherapeutic treatment ineffective so that new drugs must be administered in its place. To remedy this occurrence, current research is focusing on the genetic level to terminate the disease and to avoid the damaging side effects and development of drug resistance. One avenue of research focuses on antisense oligonucleotides to target the oncogenes, or cancer causing genes, in a specific fashion to completely inhibit the expression of the oncogenes.

Antisense Theory

Antisense oligonucleotides are polymers of nucleic acids, which can vary from 12-25 base pairs in length, and which are sequence specific and bind to the target mRNA or DNA through complementary hydrogen bonding. Antisense theory proposes that oligonucleotides, or oligos, recognize specific sequences of mRNA or DNA and bind to them, thus preventing translation or transcription of a gene. Through the binding of an oligo to an mRNA that translates an essential protein for cancer growth, the action of the protein is terminated because the product, an oncoprotein, is never formed (1). In the 1970s, Zamecnik and Stephenson were the first to show that oligos complementary to Rous Sarcoma Virus (RSV) mRNA were able to inhibit viral replication in a specific way. Some of the RSV infected cultured chicken cells absorbed a significant concentration of antisense oligo to bind with viral mRNA in a specific way to inhibit

transformation to the malignant phenotype (2). It was proposed that the oligo hybridized to the mRNA so that the ribosome was unable to bind the mRNA for protein synthesis.

Early antisense oligos were natural, short sequences of DNA synthesized in the same form that is found in cell nuclei. The DNA oligos consisted of a phosphate-sugar backbone, with phosphodiester bonds joining the sugars. The phosphodiester bond contains two non-bridging oxygens, one of which contributes to the negative charge of the DNA molecule. No chemical modifications were made to these oligos; they are referred to as PO2s, or phosphodiesters (Figure 1). As such, the unmodified phosphodiester oligos were subject to nuclease degradation as any foreign DNA is in a cell, leaving only a short time for the oligos to bind to mRNA before being subject to cellular nucleases.

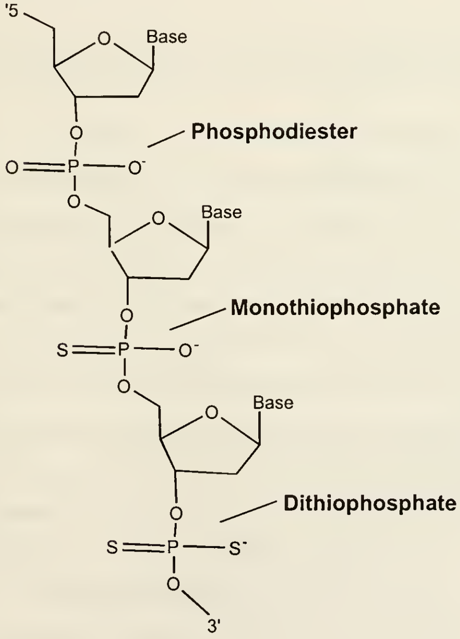
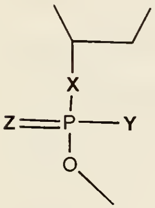


Figure 1. Modifications to the phosphate-sugar backbone of the antisense DNA molecule, including the phosphodiester, monothiophosphate, and dithiophosphate.

A solution to the nuclease digestion of PO2s was to modify one or more parts of the oligo structure. An oligo that is capable of antisense inhibition of gene expression must possess resistance to nuclease degradation, efficient uptake into the desired cells, an

affinity for the targeted mRNA, specific recognition of the targeted mRNA, and a mechanism of inhibition (4). To achieve these requirements, several attempts at modifications have been made. Methylphosphonates, phosphotriesters, phosphoramidates, and monothiophosphates are examples of modifications of the phosphate-sugar backbone of antisense DNA (4) (Figure 2).



	X	Y	Z
Methylphosphonate	O	CH_3	O
Phosphotriester	O	OR	O
Phosphoramidate	NH	O^-	O

Figure 2. Modifications to the sugar-phosphate backbone of DNA. The monothiophosphate modification is shown in Figure 1. Note: R is any organic group that is added.

The most studied of these modifications, and one object of this research, are the monothiophosphates, or POS modifications (Figure 1). POS oligos are modified through the addition of one sulfur atom in the place of one non-bridging oxygen atom in the phosphodiester backbone. The POS modification has advantages and disadvantages. Sulfur exhibits properties similar to those of oxygen, thus it is an ideal substitution because it alters some properties of the unmodified oligos, such as susceptibility to nucleases, while maintaining structural integrity and biocompatibility. The sulfur modification supplies resistance to nuclease degradation, and it also activates RNase H (4). RNase H is an enzyme for which the mRNA/POS oligo complex serves as a substrate; the RNA is hydrolyzed by the RNase H and the POS oligo is released to hybridize to a new target mRNA molecule (4).

Unwanted properties do exist for POS oligos, so they are not the ultimate choice for antisense treatments. The addition of one sulfur atom produces a chiral center around phosphorus, and thus diastereomers are formed. The diastereomers obtained synthetically have shown decreased hybridization efficiency to the oligonucleotide specified site compared to achiral molecules (18). Also, the POS oligonucleotide diastereomers offer different nuclease stability and variable stability in forming duplexes with RNA (18). It

is unknown yet whether a particular diastereomer is more effective than another because of the varying properties that have been demonstrated. The number of diastereomers that can be formed equals 2^n, where n is the number of phosphodiester bonds in the oligo. A 15 base sequence has 14 phosphodiester bonds, so there are 2^{14}, or 16,384, diastereomers that may result during synthesis. It would be nearly impossible to know which diastereomer is most effective, let alone separate the most effective one from the others.

A more recent modification, and perhaps a more promising one, is the replacement of both non-bridging oxygens of the DNA backbone with sulfur atoms, to form dithiophosphates, or PS2s (Figure 1). Such a modification eliminates the chiral center of the POS but maintains the resistance to nuclease degradation and the activation of RNase H. Sulfur atoms should not be substituted for both non-bridging oxygen positions in the backbone of a PS2 at each base position, as in the modification of the studied POS (see Figure 11), because it causes instability due to decreased hybridization affinity (18). Ghosh showed that the melting temperature of a 17-mer all phosphorodithioate (doubly substituted at all positions) was 17°C lower than the PO2 and 11°C lower than the POS (18). This shows a decreased stability with increasing sulfur substitution, so that there is a 1° depression in melting temperature for each modified base (18). It has been proposed by Cummins and co-workers that the minimal amount of dithio modification that will allow for all of the beneficial effects of the antisense oligonucleotide should be used (19), thus preventing toxicity and instability as much as possible. In this research, the dithio modifications were minimized, so that there are a maximum of three positions of double sulfur substitution (Figure 11).

In addition to the sulfur modifications being studied in this research, there are numerous other modifications to antisense oligonucleotides that are under scrutiny. Uniformly modified N3' → P5' phosphoramidates have demonstrated a high binding specificity for RNA (31). They have also shown resistance to nuclease degradation, but there is no evidence of the direction of RNase H activity (19). This modification consists of a 3' amino group substituted for a 3' hydroxyl group in the 2' deoxyribose ring of the DNA (Figure 3).

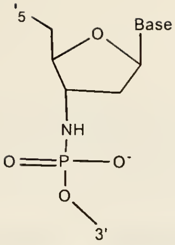


Figure 3. Phosphoramidate modification (31).

Skorski and co-workers used a 15-mer anti-*myc* phophoramidate oligonucleotide to show that it was almost ten times as potent in antisense sequence-specific activity as the anti-*myc* phosphorothioate (POS) oligo (31). The phosphoramidates possess different physicochemical properties, which may affect their uptake by cells compared to the endocytosis mechanism of uptake exhibited for phosphorothioates. This hypothesis is supported by the results that the phosphoramidates were found in higher intracellular concentration than the phosphorothioates (31).

Most recently, "second generation" antisense oligonucleotides, such as mixed-backbone oligonucleotides, have shown improved antisense properties over POS oligonucleotides (20). The mixed-backbone oligos contained POS segments at the 3' and 5' ends and a modified oligodeoxynucleotide analog, such as a methylphosphonate (Figure 2), or oligoribonucleotide analog, for example a 2'-O-methyl group, in the internal region of the oligonucleotide (Figure 4).

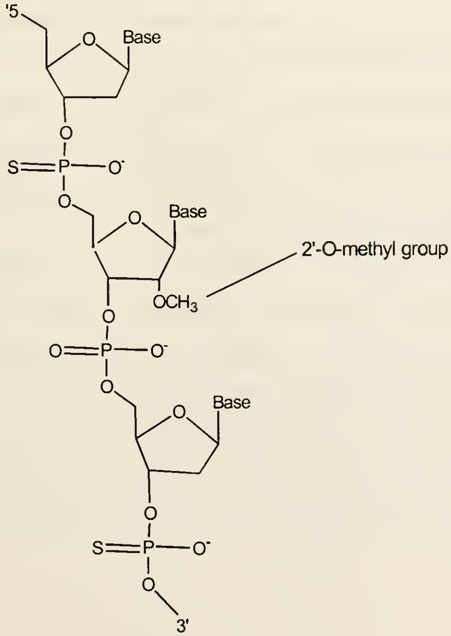


Figure 4. An example of a mixed-backbone oligonucleotide with an oligoribonucleotide analog, a 2'-O-methyl group, in the internal region and POS modifications on the 5' and 3' ends.

The idea of this type of oligonucleotide modification was to reduce the polyanionic effect of the sulfur modification for POS and PS2, a result of the negative charge at every phosphorus atom in the backbone of the oligo. The polyanionic effect stimulates the immune system in a sequence non-specific nature (20). The modification attempts to increase RNA affinity and RNase H activity, which would cause RNase H to be more active at degrading the targeted mRNA. Results of this research showed that oligos with a central mixed backbone modification, described above as a methylphosphonate or a 2'-O-methyl group, and flanking POS segments were more stable, showed more RNase H activity, and had less impact on immune stimulation (20).

Another mixed-backbone modification has been studied by Kandimalla and co-workers, that of a limited number of 2'-5' ribonucleosides within a core of a 3'-5' deoxyribonucleotide that still allows for sulfur modification of the non-bridging oxygens in the backbone (21). This modified oligo with a POS sulfur backbone bound target strands of DNA and RNA and showed greater stability against nucleases than its PO2 counterpart. It was also found that 2'-5' linkages are more stable than 3'-5' linkages (21).

Synthesis of Oligonucleotides

The synthesis of antisense oligonucleotides involves several procedures, including extensive organic syntheses of some reagents and computer-controlled syntheses of the oligonucleotides themselves. The oligonucleotides are synthesized through a series of steps, using a solid phase phosphoramidite method (22).

Oligonucleotides consist of several deoxyribonucleotides covalently bonded as a result of condensation reactions between the 5' hydroxyl group of one nucleotide and the 3' phosphate group of the next nucleotide. During synthesis, it is necessary to protect other reactive groups on the nucleotides, such as the primary amino groups, hydroxyl groups, and 5' hydroxyl groups, so that they do not participate in side reactions during the synthesis of the oligonucleotides. Protection is achieved by adding either temporary or permanent hydrophobic groups, so that side reactions to form unwanted products are prevented and so that the oligo will be soluble in the organic solvents used in synthesis.

Permanent protecting phenoxyacetyl groups (PAC) are placed on the primary amino groups of the bases. β-cyanoethyl groups permanently protect the hydroxyl groups on the phosphorus of the oligo backbone. These permanent groups remain during the entire synthesis and are removed only after synthesis is complete, during a manual deprotection procedure (22, 33). Temporary protecting groups remain in place only during a step of synthesis in order to prevent any side reactions during that particular step. The 5' hydroxyl group on the nucleotide is protected using a 4,4'-dimethoxytrityl group (DMTr), which is easily removed with acid following each cycle in synthesis. Another protecting group is the diisopropylamide that is attached to the phosphorus; it is a good leaving group that is removed during coupling.

A phosphoramidite is a fully protected nucleotide ready to be used in synthesis of an oligonucleotide; amidites of the four bases, A, T, C, and G, are used. The structure of A is shown in Figure 5.

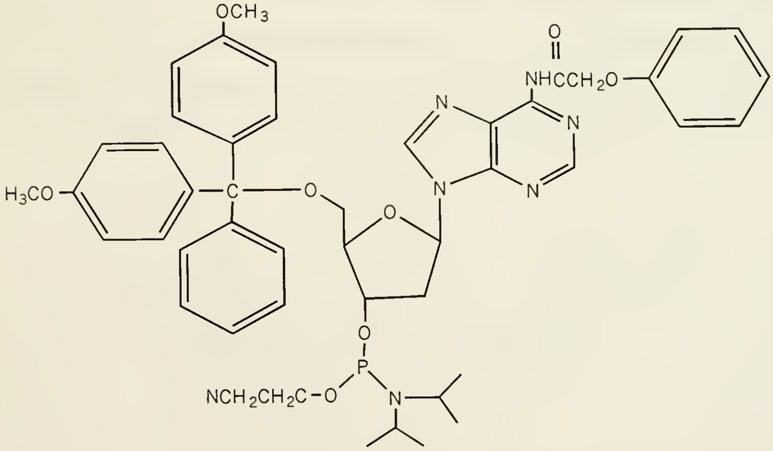


Figure 5. Structure of a fully protected adenine phosphoramidite. The 5' oxygen is temporarily protected with a dimethoxytrityl group. The base is permanently protected at the primary amine with a phenoxyacetyl group. The phosphate group is protected at oxygen permanently with a β-cyanoethyl group. The phosphorus also has a diisopropylamide leaving group attached to the site where coupling occurs (22).

The four phosphoramidites are used in the synthesis of phosphodiester oligonucleotides (PO2), monothiophosphate oligonucleotides (POS), and dithiophosphate oligonucleotides (PS2). An additional reagent is needed during the synthesis of the POS

and PS2 oligos, the antisense, or Beaucage, reagent (Figure 6). The Beaucage reagent, 3H-1,2-benzodithiol-3-one 1,1-dioxide, is a sulfur-transfer reagent that sulfurizes the phosphotriester backbone at a non-bridging position with sulfur instead of oxygen, leaving phosphorus in its more stable penta-valent state (32) (See Figure 9).

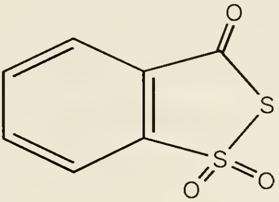


Figure 6. 3H-1,2-benzodithiol-3-one 1,1-dioxide, Beaucage reagent, used in the synthesis of phosphorothioate and phosphorodithioate oligonucleotides.

The synthesis of PS2 oligonucleotides uses the same general cycle of steps as in the synthesis of PO2 and POS oligonucleotides. However, a phosphorothioamidite (Figure 7), produced through organic synthesis, is added to the Gene Assembler as an additional reagent, to be used in the place of a phosphoramidite, depending on which backbone position is to be substituted with two sulfur atoms. The phosphorothioamidite is added to the oligo sequence through a custom method on the Gene Assembler and then sulfurized with the Beaucage reagent to give the dithiophosphate oligonucleotide.

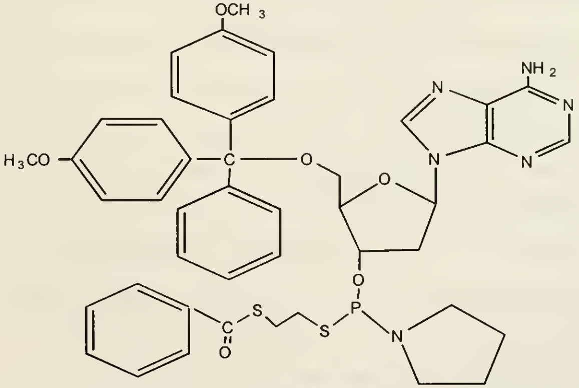


Figure 7. Phosphorothioamidite A, used in the synthesis of the PS2 oligonucleotides so that at the designated base position, there is a double sulfur substitution (32). The 5' oxygen is temporarily protected with a dimethoxytrityl group. The base is permanently protected with a phenoxyacetyl group. The phosphate bonded to sulfur is permanently protected with a β-thiobenzoylethyl group, along with a pyrrilidine-group. Phosphorothioamidite T has the same structure in regards to protecting groups but the base structure is different.

A solid phase support is used to synthesize the oligos. The sequence is elongated as each base is added on to the support. Once synthesis is complete, the oligonucleotide can be cleaved from the support for further use. The support must have the following features to be effective: insoluble in solvents and reagents, inert towards the solvents and reagents, macroporous to give access to reagents, large surface area, rigid to withstand solvent pressure, and capable of bearing functional groups to begin synthesis (22). The solid support, consisting of polystyrene particles, is derivatized to contain a primary amine group to which subsequent bases are added. It is necessary to remember that the base attached to the support is the final base in the desired sequence because synthesis follows a 3' to 5' direction, compared to the biosynthesis of DNA in the 5'to 3' direction. The functional primary amine group is protected as a β-cyanoethyl phosphoramidite and is attached to the polystyrene support via a succinate ester bridge. The bridge not only prevents steric hindrance, but also allows for simple cleavage from the support upon completion of synthesis (22).

There are four steps in one cycle of antisense oligonucleotide synthesis. The following is a description of the steps in the first cycle of synthesis, when the second base is added on to the derivatized support (Figure 8) (22). During detritylation, the 4,4'-dimethoxytrityl group (DMTr) group is removed from the 5'-hydroxyl to provide a reactive site for the coupling step. The released DMTr has a bright orange color, so that the absorbance at 436 nm can be measured to indicate the coupling efficiency of each step; coupling efficiency merely indicates the relative number of sites available for coupling. A dichloroethane wash removes excess reagents before the next step in synthesis. Coupling involves a condensation reaction between two nucleotide units. A phosphoramidite, activated by tetrazole, is added to the reactive 5'-hydroxyl group. After coupling occurs, excess reagents are washed off the support with acetonitrile. The third step is capping, in which all unreacted 5'-hydroxyl groups are capped with an acetyl group to prevent oligonucleotides of incorrect sequence from forming during the rest of the synthesis. A wash of acetonitrile follows to remove excess capping reagents. The final step in a cycle of synthesis is oxidation or sulfurization. The phosphite triester (or thiophosphite triester, in the case of a PS2 synthesis) that is formed during synthesis is oxidized with iodine or sulfurized with the Beaucage reagent (Figure 9) to form the more

stable penta-valent oxidation state. The reaction cycle is concluded with an acetonitrile wash to remove oxidation reagents.

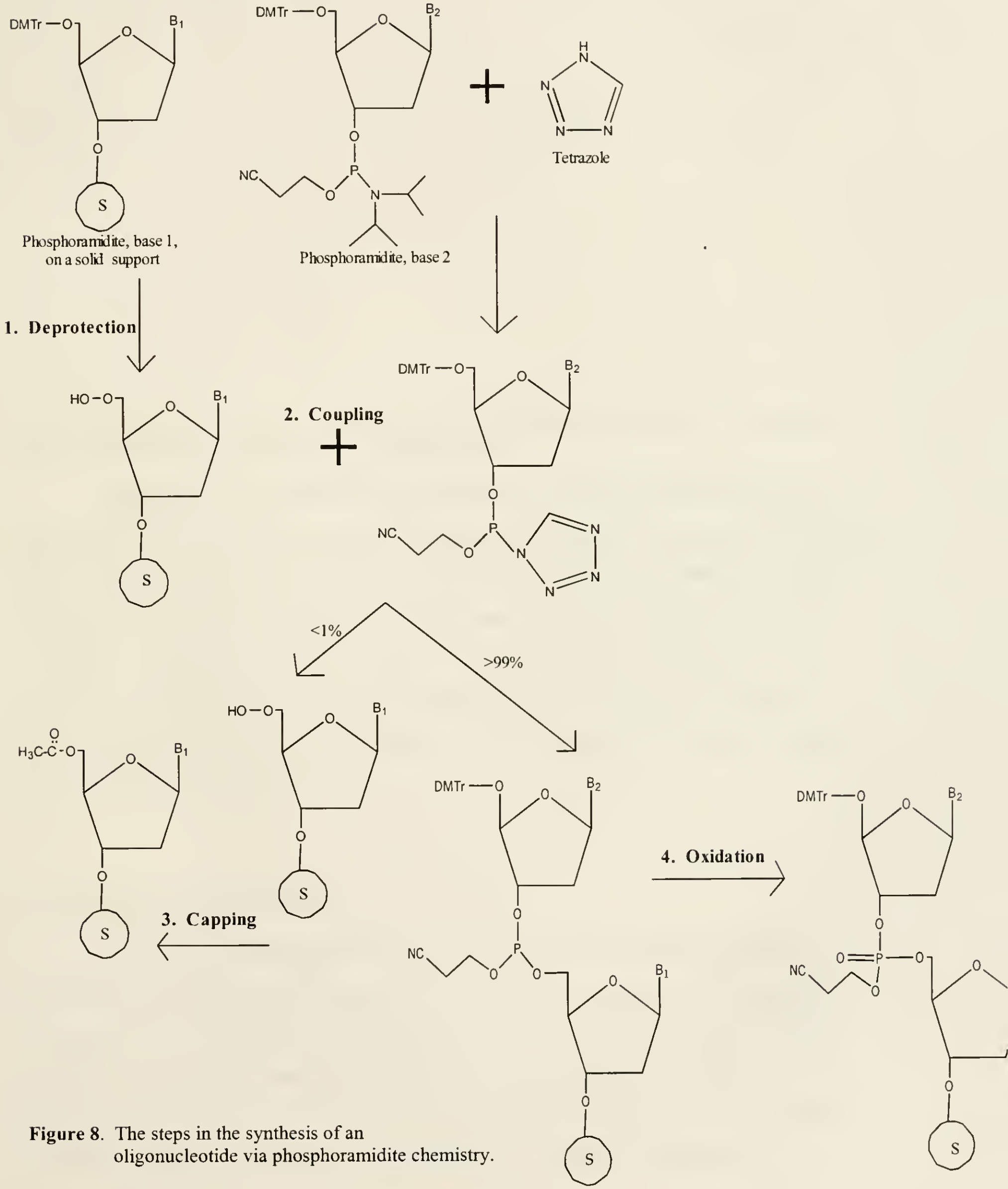


Figure 8. The steps in the synthesis of an oligonucleotide via phosphoramidite chemistry.

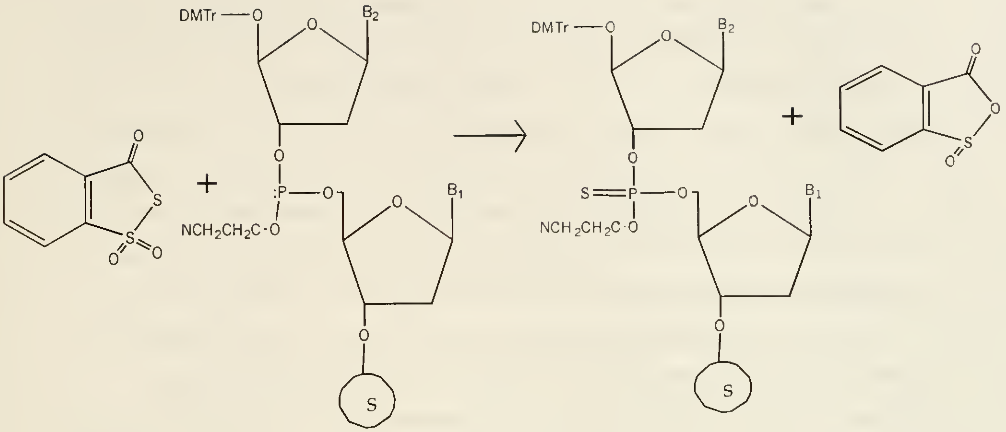


Figure 9. Sulfurization with the Beaucage reagent to give a POS oligonucleotide (32). The Beaucage reagent is also used in the synthesis of PS2 oligonucleotides.

Synthesis proceeds through this cycle of steps for each phosphoramidite that is added to the oligonucleotide. During the final cycle of steps, the dimethoxytrityl (DMTr) group is not removed from the terminal 5' base; this allows for Reversed Phase HPLC separation from failure sequences that do not possess the DMTr group. Once synthesis is complete, the oligo must be cleaved from the support and the permanent protecting groups (β-cyanoethyl groups and phenoxyacetyl groups for PO2 and POS oligos; β-thiobenzoylethyl and phenoxyacetyl groups for PS2 oligos) removed before the oligos can be purified with HPLC.

Purification of Oligonucleotides

Once antisense oligonucleotides have been synthesized and deprotected, it is necessary that they be purified before use in cell culture experiments. Reverse-phase high performance liquid chromatography (RP-HPLC) is used for this purpose. High performance liquid chromatography applies high pressure through a column to force separation of a liquid mixture. Reverse phase liquid chromatography theory holds that the mobile phase is polar and the stationary phase is non-polar. Oligonucleotides are

negatively charged polymeric anions, also called polyphosphates or polyanions, so they will interact with the two phases based on their charge and hydrophobic moieties along the backbone (23). The synthesized oligos have a DMTr group on the terminal base of the sequence. Therefore, the full length oligo would be retained in the non-polar column (stationary phase) longer than the failure sequences, which are largely attracted to the polar mobile phase because of their charge and lack of a DMTr group. The HPLC column used in this research was made of a poly(styrene-divinyl)benzene polymer with 100 angstrom pore size. It allows for greater recovery of the protected oligonucleotides that are purified with it. It is also much more stable than silica-based columns (34).

The mobile phase that is most commonly used for separation of oligos in reverse phase chromatography is triethylammonium acetate, pH 8.0 (TEAA). TEAA is used in a gradient with acetonitrile, a non-polar solvent, to allow for a gradual change in solvent composition, which in turn allows the oligos to separate. Because the full-length oligonucleotide has a large non-polar group, DMTr, on its terminal base, it will be released from the non-polar column when the concentration of acetonitrile in the gradient has increased sufficiently. The release of material from the HPLC column is detected by ultraviolet (UV) absorbance at 260 nm, so that the relative retention time of the oligo can be determined. The length, sequence, and extent of sulfur modification of the oligonucleotides affect their retention times. The purified oligo is collected over its characteristic retention time, as verified by its absorbance value. An example of the chromatograph that results from purification using HPLC can be seen in Figure 10.

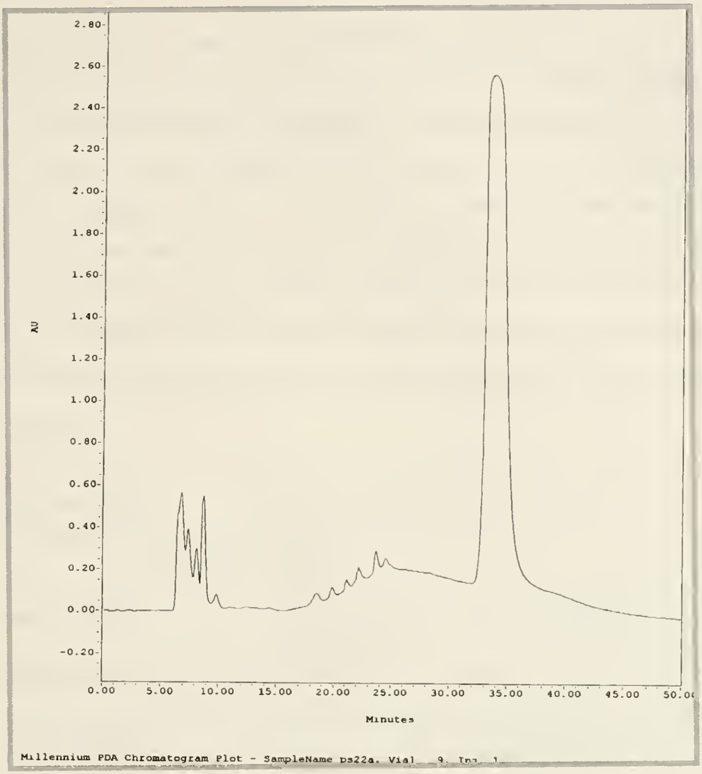


Figure 10. Chromatograph of a PS2-2 oligonucleotide (Figure 11 shows the sequence). The first peaks, between 7 and 9 minutes, are failure sequences. The second peak is the antisense oligonucleotide, at 34 minutes; this sample was collected from 33 minutes to 36 minutes, over the entire peak. Note the difference in absorbance values of the failure sequences (0.55 AU) and the oligo (2.6 AU); this indicates that there was much more oligo than failure sequences synthesized on the Gene Assembler®.

HL-60 Cells and the *myc* Oncogene

The purpose of my junior honors research with the Biology Department of Sweet Briar College was to compare the effects of anti-*myc* PO2s and modified POSs on a human leukemia cell line; this year, the research was extended to include PS2 oligos. The sequence of the antisense oligo is 15 bases in length and is complementary to the *myc* oncogene, hence the name anti-*myc*. The *c-myc* gene sequence is given in Figure 11, along with the anti-*myc* sequences and their modifications as POS and PS2 as well as two

scrambled control (POSC) sequences and modifications. Both antisense oligos and scrambled control oligos were studied in order to explore if the oligos did indeed act in a specific antisense mechanism. The scrambled control serves to determine whether the action of the oligo is sequence specific or non-specific; it has the same base composition as the antisense oligo, but the bases are randomly scrambled (5). There are two scrambled control sequences in the series of oligos studied. In order to accommodate for simpler modifications of the PS2 scrambled controls, the location of two bases was switched. A POS modification of the PS2 scrambled control was synthesized, so there were two POS scrambled controls to serve as further comparison of specific versus non-specific effects.

c-*myc* 3'TTG CAA CTC CCC ATG 5'

Anti-*myc* PO2 5'AAC GTT GAG GGG CAT 3'
Anti-*myc* PO2C 5'CTG AAG GTG CAT GAG 3'
(scrambled control)

Anti-*myc* POS 5'AxAxCx GxTxTx GxAxGx GxGxGx CxAxTx 3'
(x = POS modification)
Anti-*myc* POSC 5'CxTxGx AxAxGx GxTxGx CxAxTx GxAxGx 3'
(scrambled control)
Anti-*myc* POSC2 5'CxTxGx AxAxGx GxTxGx CxAxAx GxTxGx 3'
(scrambled control, same sequence as PS2 scrambled control)

Anti-*myc* PS2-1 5' AAC GTT GAyG GGG CAT 3'
(y = PS2 modification)
Anti-*myc* PS2-2 5' AAyC GTT GAG GGG CAyT 3'
Anti-*myc* PS2-3 5' AAyC GTT GAyG GGG CAyT 3'

Anti-*myc* PS2C-1 5' CTG AAG GTyG CAA GTG 3'
(PS2 scrambled control)
Anti-*myc* PS2C-2 5' CTyG AAG GTG CAA GTyG 3'
Anti-*myc* PS2C-3 5' CTyG AAG GTyG CAA GTyG 3'

Figure 11. The c-*myc* gene sequence and the anti-*myc* antisense oligonucleotides, with their modifications indicated as PO2, POS, and PS2. The c-*myc* gene sequence was obtained from Reference (8) and (17). The scrambled control sequence (PO2C and POSC) differs in one codon from the sequence in Reference (5) because of the supply of amidites for synthesis. Note that the PS2 modifications for the anti-myc sequence and the scrambled control sequence are modified in the same locations – middle, end, and both end and middle – but the base that is modified is different. The POSC2 sequence changed the position of two bases to allow for simpler modification and was a result of reagent availability.

HL-60 is a human promyelocytic leukemia cell line derived in 1977 from the peripheral blood leukocytes of a patient with acute promyelocytic leukemia (7). Leukemias arise in blood-forming cells of the bone marrow. It is typical for leukemias to fail in normal differentiation while retaining their ability to proliferate, thus continuing to reproduce indefinitely. Normal blood cells, in comparison, will differentiate into mature red blood cells (erythrocytes), lymphocytes, macrophages, and granulocytes (9). Once these cells have matured, cell growth serves maintenance purposes only. These cultured suspension cells exhibit aneuploidy, as they have only 44 chromosomes (7). HL-60 cells are arrested in the promyelocytic stage of myeloid development; they possess proliferative capacity but are unable to differentiate in the absence of inducing agents (8).

HL-60 cells show high levels of c-*myc* RNA, amplified 8-30 fold compared to normal cells. The high expression of c-*myc* in the cells occurs in direct association with an activated N-*ras* gene and the deletion of the nuclear p53 tumor suppressor gene (8). The common cause of promyelocytic leukemias is the reciprocal translocation between chromosomes 15 and 17, however, HL-60 cells do not display such abnormalities in their chromosomes (10). Murray and co-workers suggest that an alteration independent of this translocation causes the amplification of c-*myc* in HL-60 cells (11).

The *myc* oncogene is a cause of cancer development because it permits the overexpression of the cellular *myc* gene (c-*myc*), the proto-oncogene. The function of c-*myc* in normal cells is indicated by its gene product; c-*myc*, when activated in response to growth factor stimulation, produces a nuclear protein that is implicated in the control of normal cell division (9, 12). When c-*myc* is amplified, as it is in HL-60 cells (10), it overexpresses this growth regulating protein so that the cells continue proliferating even in the absence of growth stimulating factors. If the expression of c-*myc* can be inhibited, for example with antisense oligos, cell growth can be decreased. Holt, Redner, and Nienhuis showed levels of c-*myc* protein to decrease 50-80% in HL-60 cells upon treatment with antisense oligos (8). These researchers used PO2s, and noted that the oligos were able to enter the cells to form a 15 base pair double-stranded duplex with the c-*myc* RNA, which in turn inhibited cell growth through decreased c-*myc* expression and induction of cell differentiation (8). If the promyelocytic cells cease proliferation in the

early stages of myeloid development and are induced to differentiate, the growth of those cells is thus regulated.

Cationic Liposome Transfection

A difficulty in using antisense oligos arises when the cells must take up the DNA. Random uptake by the cells is not dependable nor is it highly likely because the antisense oligos possess a large net negative charge. It is difficult for a 15 base pair, negatively charged oligo to pass through the plasma membrane of the cell unaided. Experiments in the past have simply supplemented the media in which the cells grow with antisense oligo and allowed the random uptake into the cells, and some antisense action has been observed (8). However, more efficient uptake of antisense oligos is desirable. Cationic lipids show promise for forming a complex with antisense oligos that will allow uptake by cells and subsequent expression of the oligo in the cell (13, 14, 15, 16). The lipid-DNA complex is able to bind to cultured cells and fuse with the plasma membrane. The liposome formulation utilized in this research is known commercially as Lipofectin™; it is a 1:1 (w/w) combination of the cationic lipid, DOTMA (N-[1-(2,3-dioleyloxy)propyl]-n,n,n-trimethylammonium chloride) (Figure 12), and the neutral lipid, DOPE (dioleoyl phosphotidylethanolamine), in an aqueous solution that forms 250 nm liposomes (15, 16). The antisense oligos are encapsulated in the Lipofectin™ through the spontaneous formation of multilamellar liposomes; the DNA is in the center of the vesicles, bound through ionic interaction to the positively charged group on the DOTMA molecule (13) (Figure 12).

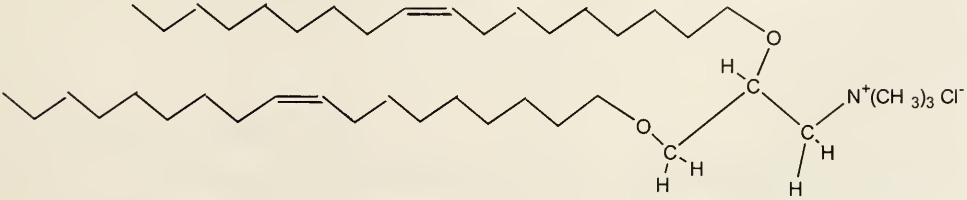


Figure 12. Structure of (N-[1-(2,3-dioleyloxy)propyl]-n,n,n-trimethylammonium chloride), DOTMA. Note the long hydrophobic side chains that will associate to form the outer surface of the liposome, and the positively charged group that will interact with the negatively charged phosphate backbone of the oligonucleotide (13).

Lipofectin™ facilitates uptake of oligos into cells by effectively neutralizing the negative charge of the oligonucleotide (13). This increases the opportunity for antisense activity by increasing the amount of oligo available within the cell to interact with the target mRNA. Lipofectin™ may allow the distribution of the oligonucleotides in the cell to concentrate in the nucleus, in the vicinity of the target mRNA, through an unknown mechanism (16). Evidence for these results is shown in FITC labeling studies and kinetic experiments with the cationic liposome (16).

A difficulty in using cationic liposomes in association with oligos for cell transfection is that serum, both *in vitro* and *in vivo*, has been shown to interfere with the delivery of oligos to cells (24). There are two proposed actions of inhibition by serum components. The components may coat the liposome complex, thus resulting in either an increased complex size which is unable to be endocytosed by the cell or a sterically hindered form that is unable to release the oligo within the cell. The serum components may also cause the liposome/oligo complex to dissociate, so that the oligo is released before it even reaches the target cells (24).

A recent development in oligonucleotide transfection takes into account the destabilizing properties of serum and provides a carrier molecule that may be more effective in medical use (25). Meyer and co-workers modified cationic liposomes with a phospholipid derivative of polyethylene glycol (PEG-PE) to achieve enhanced oligo uptake, prevention of liposome aggregation from serum component action, and increased complex stability (25). These new molecules also allowed for a higher amount of oligo to be encapsulated than when just a cationic liposome was used, so that more oligo is available within the cell for antisense activity.

Mechanism of Oligo Action

Once the Lipofectin™ has efficiently delivered the oligonucleotide to the cell, the oligo is free to act to inhibit the expression of the oncogene. The mechanism of action within the cell is not known exactly, but there are four proposed mechanisms described here. It is possible that the contiguous tetraguanylate (4G) tract within the anti-*myc*

sequence (Figure 11) forms a complex structure that inhibits cell proliferation in a non-specific fashion (27, 29). Another possibility is the antisense specific binding of the oligo to the major groove of DNA to form a triplex that competes with DNA binding proteins for transcription initiation (30). It is also likely that the oligo could bind to mRNA to stop the expression of protein by inhibiting ribosome-mRNA association; the oligo/mRNA complex also serves as a substrate for RNase H (26, 28).

A contiguous tetraguanylate sequence within the anti-*myc* sequence is known to form a higher order structure, such as a tetraplex (27) (Figure 13). This structure may interfere with the base pairing of the oligo with the target mRNA, so the effects on cell growth could be a result of a non-antisense mechanism. It is possible that the oligo with four guanines binds to serum proteins in a structure-specific fashion, creating a protein-oligo interaction that decreases cell growth (37). However, it has been demonstrated that the tetraguanylate tract does not always exert a non-specific effect on cell growth; Basu and co-workers found that no tetraplex structure formed at 37°C under physiologically relevant conditions (27). Furthermore, complex structure formation was not facilitated nor stabilized in the presence of serum. Several factors influence the formation of these higher order structures, including the presence of ions, the concentration of DNA and ions, the length of the guanine-rich segment, and the extent of complementarity within the sequence (27). Tetraplex structure can be formed by tetraguanylate sequences in oligos, but it is not highly likely in the concentrations of oligo supplied to cells nor in the cellular conditions to which the oligos are subject (27).

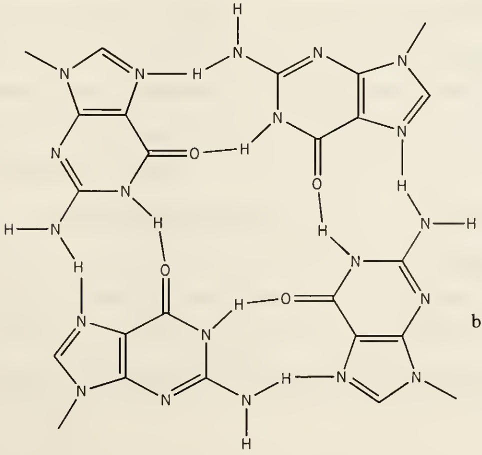


Figure 13. Theorized structure of a tetraplex in a contiguous tetraguanylate tract in DNA. The higher order structure may be parallel or anti-parallel and forms hydrogen bonds between each of the four guanine bases (35).

Saijo and co-workers also propose a second mechanism of oligo action involving the contiguous four-guanosine sequence that is present in the anti-*myc* gene sequence (29). Growth inhibition of human lung cancer cells by anti-*myc* was antisense sequence non-specific and depended on the tetraplex structure instead when the oligo was applied at high concentrations (10 μM). The tetraplex structure also caused loss of cell adhesion and changes in cell morphology (29). Some results of this study led to the conclusion that an antisense effect may be exerted at lower concentrations of oligo (1 μM), through the inhibition of c-*myc* protein expression. Specific inhibition was demonstrated when a scrambled control sequence containing a four-guanosine tract was compared to an antisense sequence that also contained a four-guanosine tract. The antisense sequence caused a greater decrease in c-*myc* expression than the scrambled control sequence (29). Therefore, the observed inhibition of cell growth may be a combination of sequence specific and sequence non-specific mechanisms. In addition to the action of the tetraguanylate tract, another non-specific mechanism has been demonstrated to contribute to the overall effect of oligos on cell growth. The polyanionic oligos may bind to proteins such as fibroblast growth factor and inhibit protein binding to cell surface receptors, thus inhibiting cell proliferation (29).

A third antisense mechanism that has been proposed for oligonucleotide action explains that the short oligo binds in a sequence specific fashion to the double stranded DNA in its major groove (30). The intermolecular triplex that is formed inhibits transcription of c-*myc* because the initiation site is blocked from binding a site-specific DNA binding protein. Both reverse transcriptase and DNA polymerase are unable to carry out their function when the triplex-forming oligo has successfully bound itself in a sequence specific manner to the major groove of the duplex DNA. The inhibition of transcription of c-*myc* would lead to a decline in cell growth because the oncogenic protein is never formed.

Holt, Redner, and Nienhuis conceived of a fourth antisense mechanism for anti-*myc* in which the oligo forms a duplex with its complementary mRNA (8). The duplex caused inhibition of *c-myc* expression and subsequent growth inhibition and cell differentiation. When the oligo binds to the mRNA to form the duplex, the ribosome is unable to bind and therefore protein synthesis is inhibited (4). In addition, the

mRNA/oligo complex serves as a substrate to activate the enzyme RNase H (4). Thus, RNase H cleaves the RNA strand, releasing the oligo in the process to allow further binding to more mRNA.

Despite the lack of a definite mechanism for oligonucleotide action, research has shown that cancer cell proliferation can be inhibited through the addition of antisense oligonucleotides. Whether the mechanism is sequence specific, sequence non-specific, or a combination of both, oligos have proven to be effective. Modifications of oligos are now a focus in research, to develop an antisense oligo that can carry out the desired action, inhibiting the growth of cancer cells, with the least amount of difficulty, such as nuclease degradation or diastereomer formation.

Phosphorodithioate antisense oligonucleotides are a possible solution for cancer treatment because they overcome several problems of monothiophosphate oligos and they have shown in a few early studies that they are effective in inhibiting cell growth (18, 19, 28). Thus, in this research the possibility of antisense activity was explored using PS2 antisense oligonucleotides. The oligos were applied in cell culture studies to HL-60 cells to determine their effect on cell growth compared to POS and PO2 oligos.

Materials and Methods

Synthesis of Antisense DNA

The oligonucleotides were synthesized on a 0.2 μmole scale using the Pharmacia LKB Gene Assembler Special. The general steps of the synthesis were described in the Introduction. Two solid support polystyrene cartridges were purchased from Pharmacia Biotech; “T” cartridges were used for PO2, POS, and PS2 sequences and “G” cartridges were used for the scrambled control sequences (refer to Figure 11). The phosphoramidites, A, C, T, and G (Figure 5) were supplied by Pharmacia Biotech for use in the synthesis of all oligonucleotides. The phosphoramidites were dissolved in anhydrous acetonitrile according to product information to a 0.1 M concentration; flame-dried 3-angstrom molecular sieves, obtained from Sigma-Aldrich, were added to each

reagent bottle. The remaining reagents were obtained from Pharmacia Biotech: tetrazole (0.5 M, dissolved in anhydrous acetonitrile); capping A (20% N-methylimidazole in acetonitrile); capping B (2,4,6-collidine in acetonitrile and acetic anhydride); acetonitrile (dried over flame dried 3-angstrom molecular sieves for at least 24 hours); dichloroethane; detritylation (trichloroacetic acid and dichloroethane); and oxidation (iodine in acetonitrile).

POS oligo synthesis involved the use of an additional reagent, the Beaucage reagent (32), also purchased from Pharmacia Biotech (Figure 6). The Beaucage reagent concentration was 0.05 M in anhydrous acetonitrile; no molecular sieves were added.

Dithiophosphate (PS2) synthesis utilized all the reagents used for PO2 and POS synthesis. In order to modify the backbone with two sulfur atoms, a special modified phosphoramidite, a thioamidite (Figure 7), was synthesized by Dr. Jill N. Granger and Leslie Farinas (Sweet Briar College), Jennifer Swisher (Sweet Briar College), and Rebekah Cowell (Lynchburg College). The procedure followed was that of Wiesler, Marshall, and Caruthers (33). Two different thioamidites were used in the syntheses: an adenine (A) thioamidite was used for the antisense PS2 oligos (971PS2 sequences) and a thymine (T) thioamidite was used in the synthesis of the scrambled control PS2 oligos (991PS2C sequences). Each thioamidite was dissolved in anhydrous acetonitrile to make a 0.1 M solution and flame dried 3-angstrom molecular sieves were added.

Deprotection of Antisense DNA

Following synthesis, the oligos were deprotected to remove all of the protecting groups, except for the final dimethoxytrityl group, from the oligo sequence. The PO2 and POS oligos were deprotected and removed from the support cartridge with concentrated ammonium hydroxide overnight at room temperature (22). They were then desalted using a disposable NAP-10 column, containing DNA grade Sephadex G-25, purchased from Pharmacia Biotech. The NAP column is able to remove all small molecular weight materials in the DNA solution, including released protecting groups and ammonia and oligos up to ten bases in length (22). Briefly, the procedure for separation using the NAP column involves applying the sample, in a volume appropriate for the size of the column, in this case 1000 µl, to the column. The eluted sample is collected upon addition of water

to the column, which forces the sample to elute. The procedure followed for deprotection and desalting is described in detail in the Gene Assembler Manual (22). Following desalting, the PO2 and POS oligos were ready for purification by HPLC.

PS2 oligos required a different deprotection procedure since they possess different protecting groups. The kinetics of the cleavage of these groups is different because they are more labile. Instead of using concentrated ammonium hydroxide, a 1:5.7 mixture of 2M ammonia (in ethanol solution) and benzene was used to deprotect the DNA. The methods followed were exactly as described by Weisler, Marshall, and Caruthers (33). Deprotection took place at room temperature for 1 hour, then at 55°C for 15 additional hours. Toluene washed the ammoniacal ethanol/benzene mixture from the support cartridge, so that the DNA could be cleaved and absorbed into a 3:1 ultra-pure water-acetonitrile mixture. The PS2 oligos were then ready for HPLC purification.

Purification of Antisense DNA

Purification of the oligos was achieved using reversed-phase high performance liquid chromatography (HPLC) with a Waters 510 Pump and Hamilton PRP-1 (polymeric reversed phase) column (7.0 x 305 mm, 10μm particle size). Millenium 2010 Chromatography Manager Software, Version 2.00, was used to control the HPLC and analyze the oligo purification process.

For each run of the HPLC, 250 μl of DNA in solution (in either ultra-pure water for the PO2 and POS oligos or an ultra-pure water-acetonitrile mixture for the PS2 oligos) was injected. A "run" lasted 50 minutes per injection and the purified DNA was collected at a retention time of between 20 and 30 minutes (varying according to the sequence and its modifications). The gradient changed for each modification of the oligonucleotides, as is demonstrated in Tables 1, 2, and 3. The tables show ramped gradients, which means the solvents change in a linear fashion as time proceeds.

Time (min)	Flow (ml/min)	% Water	% TEAA	% Acetonitrile
0	1.0	0	80	20
5	1.0	0	80	20
15	1.0	0	70	30
30	1.0	0	70	30
40	1.0	0	80	20
50	1.0	0	80	20

Table 1. Gradient method for HPLC purification of PO2 antisense oligonucleotides.

Time (min)	Flow (ml/min)	% Water	% TEAA	% Acetonitrile
0	1.0	0	80	20
5	1.0	0	80	20
15	1.0	0	65	35
30	1.0	0	65	35
40	1.0	0	80	20
50	1.0	0	80	20

Table 2. Gradient method for HPLC purification of POS and POSC antisense oligonucleotides.

Time (min)	Flow (ml/min)	% Water	% TEAA	% Acetonitrile
0	1.0	0	80	20
5	1.0	0	80	20
15	1.0	0	60	40
30	1.0	0	60	40
40	1.0	0	80	20
50	1.0	0	80	20

Table 3. Gradient method for HPLC purification of PS2 and PS2C antisense oligonucleotides.

The failure sequences elute from the column within the first ten minutes, but the successful sequence, which has the DMTr group still attached, was released from the column later when the polarity of the gradient decreased. The DNA that was eluted was collected from the beginning of the peak until the end of the peak, as detected by absorbance at 260 nm, and stored in a siliconized flask at -20°C.

Preparation of Purified Antisense DNA for Cell Culture

Following HPLC purification, the oligos were lyophilized with an FTS Systems, Inc. Flexi-Dry μP Freeze Dryer to reduce the volume of the oligo in solution and to reconstitute the oligo to a small, more manageable and concentrated volume. The oligos were in a solution of TEAA Buffer and acetonitrile after HPLC purification; the large volume was frozen with liquid nitrogen (-190°C) before being added to the lyophilizer. The antisense oligos were reconstituted in 100 μl ultra-pure 18 MΩ water (Continental® Water Systems Corporation, Modulab® ModuPure Plus™ Reagent Grade Water System) after lyophilization. Manual detritylation, the removal of the dimethoxytrityl protecting group, was achieved by the addition of glacial acetic acid in a 4:1 volume ratio with ultra-pure water. Ether extraction removed the dimethoxytrityl groups from the solution to yield the desired unprotected oligonucleotides in ultra-pure water. The absorbance at 260 nm was measured (Beckman Instruments DU® 640 Spectrophotometer) in order to calculate DNA concentration. DNA concentration was calculated as a function of absorbance, a dilution factor, and a constant, 33 μg/ml (Equation 1).

$$\text{Absorbance}_{260} \times 33\ \mu\text{g/ml} \times \text{dilution factor} = \text{DNA concentration}$$

Equation 1. Used to calculate DNA concentration when absorbance value is known.

Ethanol precipitation, using NaOAc (final concentration of 7.2×10^{-2} M) and $MgCl_2$ (final concentration of 2.4×10^{-3} M), was the final step in preparation. In order to have all oligos of the same final concentration, 1400 μg/ml, the appropriate amount of sterile TE was added to the ethanol precipitated oligos.

Cell Culture of HL-60 Cells

The HL-60 cell line was purchased from the American Type Culture Collection of Rockville, MD. Cells were maintained in RPMI 1640 medium supplemented with 20% heat inactivated fetal bovine serum (both obtained from Gibco BRL). Heat inactivation of the serum was carried out after purchase at 56 °C for 30 minutes. Fresh medium was

added to cells every other day, to increase the culture volume by approximately 30%. The suspension cells were grown in a controlled environment incubator (VWR Scientific, Model 2675) at 37 °C, 80% humidity and 5% CO_2.

Lipofectin™ Transfection of Antisense Oligonucleotides

Lipofectin™ was obtained from Life Technologies™ for use in administering antisense oligos to HL-60 cells. The manufacturer's protocol for transient transfection of suspension cells in the presence of serum was followed (15). Each tube of cells contained 0.8 ml of cell suspension, which provided approximately 2-3 x 10^6 cells per tube. A mixture of 5 μl of Lipofectin™ and 3 μg of antisense oligo were added and liposomes were allowed to form for 15 minutes. Following liposome formation, the mixtures were added, as indicated below, to each tube of cells. Each experiment contained duplicates of the following:

- Cells only: assures cellular growth.
- Lipofectin™ plus cells: determines the toxicity, if any, of the liposome preparation.
- PO2 antisense oligo plus cells: verifies instability in the cell and thus no antisense action and serves as a control in comparison to the sulfur containing oligos for toxicity.
- PO2C plus cells: compares sequence specificity (thus, antisense action) with PO2 oligo.
- POS plus cells: determines antisense activity against c-*myc*.
- POSC plus cells: two different sequences compare the action of the POS oligo to determine whether the mode of action is antisense or non-specific as well as providing another means for comparison of sequence influence (one scrambled control corresponds to the PS2 scrambled control sequence).
- PS2-1, 2, and 3 plus cells: determines whether dithio modifications are more efficient at halting cell growth in comparison to the PO2 and POS oligos; also, verifies if one modification is superior to another.
- PS2C-1, 2, and 3 plus cells: compares to the antisense dithio sequences to show whether any effect on cell growth is due to antisense action, a non-specific action, or possibly due to sulfur toxicity.

Determination of Effects of Oligonucleotides on HL-60 Growth

In order to measure the increase or decrease of cell growth during an experiment, the method of trypan blue exclusion was utilized. Trypan blue is a dye obtained from

Gibco BRL that stains dead cells. It was added to small aliquots of cell suspension when the cells were counted. Upon addition of the dye, dead cells are stained a solid blue and they may appear rounded or shriveled up. Viable cells appear healthy, round, and are not stained by the dye. A hemacytometer, a glass slide with a small volume etched glass counting chamber, was used to quantitate cell growth. During an experiment, cells were counted every 24 hours for a total of 96 hours; cells received fresh medium after 48 hours (such volume changes were accounted for in calculations). Four cell counts were performed for each duplicate cell culture and averaged, followed by another average of the two duplicate averages for each variable. Growth curves were plotted for each experiment based on the raw data averages. A total of two experiments were performed, using the same series of anti-*myc* sequences each time. Based on the raw data averages, best fit lines were calculated and plotted as trend lines for each of the experiments' results. The second experiment was subject to a more mathematically correct analysis of the raw data, so that best fit lines were calculated using all of the raw data cell counts and not the average cell counts. This information provided a more precise view of the results of the cell experiment.

Results and Discussion

Synthesis and Purification of Antisense Oligonucleotides

Oligonucleotide Sequence	Concentration, μg/ml	Total Yield, %
971PO2	N/A	N/A
971POS	N/A	N/A
971POSC	N/A	N/A
991POSC	N/A	N/A
971PS2-1	107.65	5.44
971PS2-2	94.41	4.74
971PS2-3	58.11	14.96
991PS2C-1	51.36	11.89
991PS2C-2	70.78	14.64
991PS2C-3	63.57	13.07

Table 4. Concentrations and total % yield of select oligonucleotide sequences. Concentrations were calculated before ethanol precipitation using the absorbance values that were measured following detritylation procedures (Equation 1, dilution factor of 4). N/A indicates an undetermined concentration due to nonlinear absorbance values; % yields were not calculated for these sequences. Concentrations of all oligos after ethanol precipitation were 1400 μg/ml. Total percent yield uses the theoretical yield of 0.2 μmol synthesis from the Gene Assembler and takes into account all procedures that occur between synthesis and final ethanol precipitation. Final volumes were estimated to determine the yields of the PS2 oligos. No PO2 scrambled control (PO2C) was synthesized; 1400 μg/ml PO2C was available from previous studies.

Calculated data for the oligos that were synthesized and purified are presented in Table 4. The concentrations of the oligos were determined using Equation 1, following the detritylation procedure and before ethanol precipitation; the dilution factor was 4. The total yields of the oligonucleotides are very low based on the 0.2 μmol synthesis scale of the Gene Assembler. It is obvious that there is at least one step in the preparation of the oligos where significant product is lost. It would be useful to know which step(s) causes the loss of product in order to find a more efficient method. That could be accomplished by measuring the absorbance values after *each* step: following the deprotection/desalting procedure after synthesis, after HPLC purification, after lyophilization, and after detritylation. I propose that one of the largest losses of product occurs after lyophilization, when the oligo is resuspended in ultrapure water. It is difficult to know if the entire product has been retrieved, as it is dried to the sides of siliconized glass.

Upon completion of all syntheses and purification procedures, it was discovered that a reagent was added incorrectly to the Gene Assembler before the synthesis of the PS2 scrambled controls. Instead of a Capping B reagent being added in the correct place, a Capping A reagent was added, so that there were two Capping A bottles on the Gene Assembler. This resulted in an unpredictable amount of incorrect sequences being synthesized because the Capping B reagent contains the acetic anhydride that donates the capping group; all unreacted 5'-hydroxyl groups are capped with an acetyl group (Figure 8). Thus, during synthesis, the lack of a capping acetyl group allowed the incorporation of a small number of phosphoramidites in random order in the sequence, as failure sequences were not prevented from forming. Because this was not discovered until *after* all procedures were finished and the oligos were ready for the cell culture experiments, there was not much that could be done about the situation. It can be reasoned that because the sequences being synthesized without the Capping B solution were scrambled controls, it should not matter what the sequence is. Base composition is the only factor that is important in a scrambled control, and the composition did not vary by more than a small fraction of bases in the final sequence. If the sequences had been the anti-*myc* sequences, it would have been a problem, because the sequence would not be correct to match with the *myc* gene in the HL-60 cells. It was assumed that since the sequences that were synthesized with two Capping A reagents were scrambled controls, they should behave as other scrambled controls would because the bases in the sequences are indeed scrambled. These PS2 scrambled control sequences were used in the experiments despite the problems that occurred. The following discussions about the HL-60 cell culture experiments do not refer to the problems with the synthesis procedure, because they showed no adverse affect on the cells.

Cell Culture Experiments with Antisense DNA

An experiment consisted of the addition of Lipofectin™ and an oligo to a small tube of HL-60 cells, so that every 24 hours a cell count was performed. After each experiment a growth curve was plotted, based on the cell counts, to show the effects of the antisense oligonucleotides on the growth of the HL-60 cells. Table 5 summarizes the

effects of the oligos on cell growth by showing the percent decrease or increase in cell count. The data represented in this table is explained after the results of each individual experiment are presented.

Percent Decrease in Cell Count Compared to Cells Alone			
	Experiment #4 (Spring 1998) @ 120 hours	**Experiment #1 (Spring 1999) @ 96 hours**	**Experiment #2 (Spring 1999) @ 96 hours**
Lipofectin	22.8% +	8.5%	5.2%
PO2	2.7% +	7.1% +	7.3%
PO2C	3.6%	5.3% +	0.6%
POS	33%	17.1%	24.9%
POSC-1	19.5%	0.8% +	1.5%
POSC-2		3.6% +	2.1%
PS2-1		36.9%	51.5%
PS2-2		41.3%	49.7%
PS2-3		39.6%	52.7%
PS2C-1		3.9%	0.9% +
PS2C-2		10.8%	0.6% +
PS2C-3		1.6%+	1.8% +

Table 5. Quantified effects of oligonucleotides on HL-60 cell growth. Percentages were calculated using the trend data presented in Table 7 (Experiment #4), Table 9 (Experiment #1), and Table 11 (Experiment #2). The cells growing alone at the final time point were considered to be 100%. Thus, some cell counts were above the 100% point. + indicates a percentage increase compared to the cells alone.

Data from the Spring of 1998 is included in the results as a comparison for effects of the oligos (Table 6, Figure 14, Table 7, Figure 15). This data was preliminary because only PO2 and POS oligos were studied. The experiment shown here lasted 120 hours. Two experiments were carried out for 96 hours per experiment during the Spring of 1999. All antisense oligos and scrambled controls were included in these studies.

Table 6. Time and average cell count data for experiment 4, Spring 1998.

		Average	Cell	Count	(x 10^5)	
Time	**Cells**	**Lipofectin**	**PO2**	**PO2C**	**POS**	**POSC**
0	4.60	4.60	4.60	4.60	4.60	4.60
24	4.36	4.37	4.80	5.18	3.66	4.44
48	5.78	5.03	6.46	4.92	5.08	5.30
72	6.16	8.15	5.74	6.41	5.18	4.60
96	6.55	8.12	5.99	6.58	5.21	6.05
120	9.55	11.30	10.50	9.07	5.66	7.58

Figure 14. Growth curve for experiment 4, Spring 1998, including PO2, PO2 scrambled control, POS, and POS scrambled control oligos.

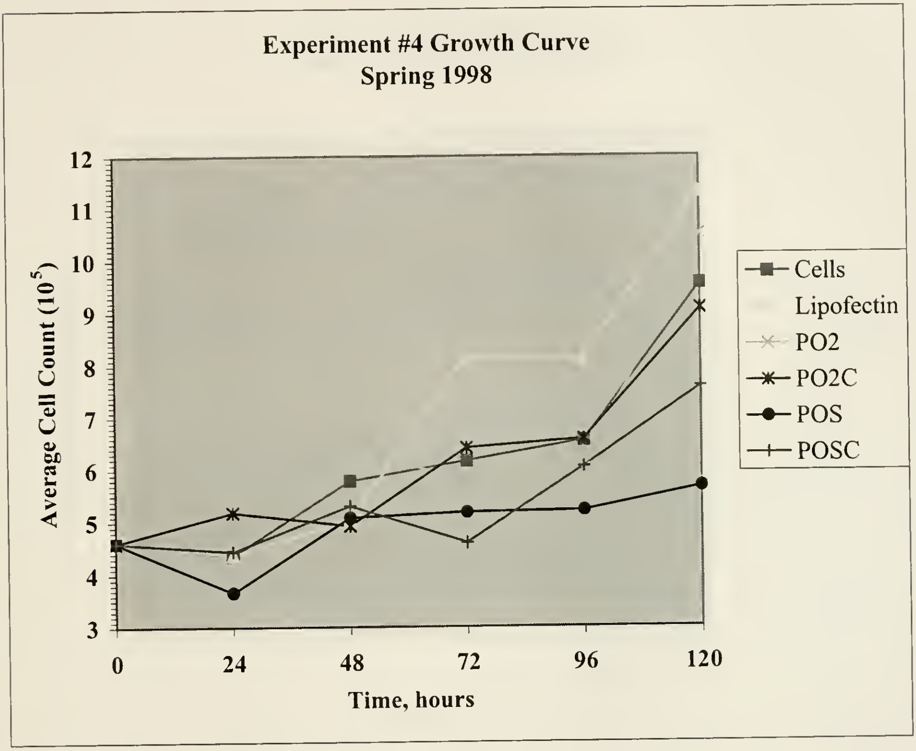
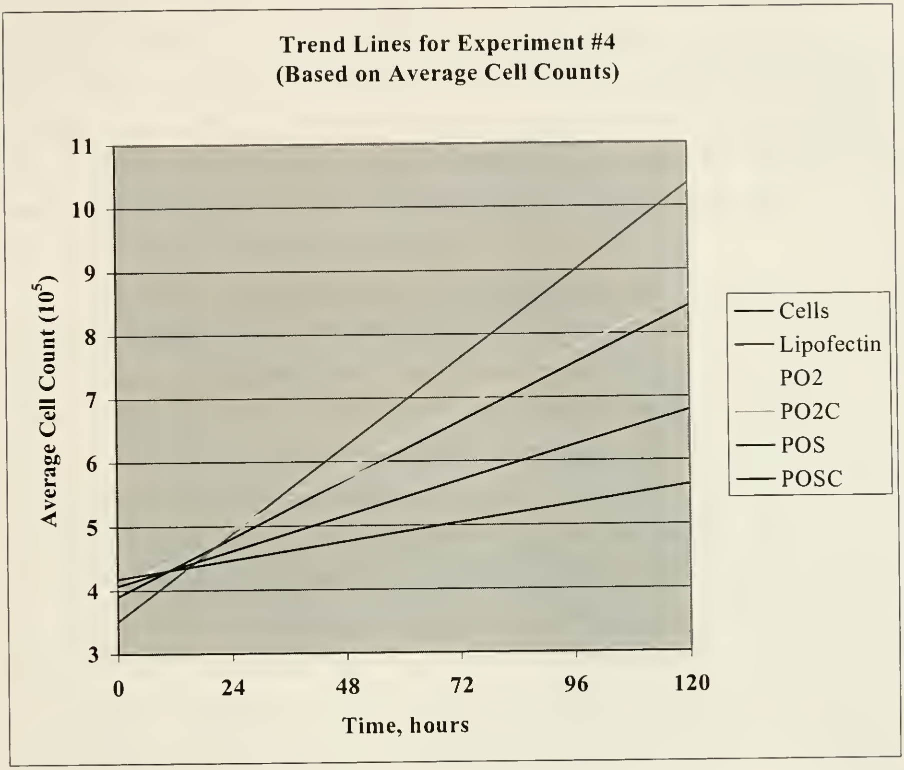


Table 7. Trend data obtained from Microsoft Excel for Experiment #4, Spring 1998. This data gives the best fit line for the average cell counts in Table 6.

		Trend Data for Experiment #4				
Time	**Cells**	**Lipofectin**	**PO2**	**PO2C**	**POS**	**POSC**
0	3.90	3.51	4.04	4.12	4.18	4.07
24	4.81	4.88	4.96	4.92	4.47	4.61
48	5.71	6.24	5.89	5.73	4.75	5.16
72	6.62	7.61	6.81	6.53	5.04	5.70
96	7.53	8.98	7.73	7.33	5.33	6.24
120	8.43	10.35	8.66	8.13	5.62	6.79

Figure 15. Trend lines for Experiment #4. This graph demonstrates more simply what Figure 14 shows.



The results of this experiment were the most satisfactory of the four experiments during the Spring of 1998. The cells alone grew normally, with a gradual increase in cell count every 24 hours. The cells and Lipofectin™ grew with only a very slight decrease at the beginning of the experiment; the cells with Lipofectin™ actually grew 22.8% more than the cells alone. The PO2 sequence did not adversely affect cell growth; there was an increase in cell count followed by a slight decrease. The final count was just slightly higher (2.7%) than the cells alone. The cells with PO2 scrambled control (PO2C) oligo grew normally, with only a slight decrease at 48 hours. Overall, the PO2C showed a 3.6% decrease in growth compared to the cells alone. The POS oligo showed marked

antisense activity; the cell count for the POS remained almost constant with no sudden growth, at 33% below the growth of the cells alone. The POS scrambled control (POSC) verified the antisense activity of POS because the cells transfected with POSC showed some cell growth. However, the POSC had a 19.5% decrease in cell growth compared to the cells alone, and this is a significant amount. It is possible that this decrease in growth could be attributed to sulfur toxicity. But, the 33% decrease caused by the POS would have to then take into account sulfur toxicity as well as an antisense mechanism in explaining its effects on decreasing cell growth.

The sudden increases and decreases in cell growth are easily attributed to beginner's experimental error. An effort was made to correct the variations by calculating and graphing trend lines for each experiment. It is likely that the cell has acted upon the foreign PO2 and PO2C anti-*myc* oligos through nuclease degradation. The POS sequence may not be as effective as the PS2s have the potential of being, since POS oligos are chiral and form diastereomers. Nonetheless, the POS anti-*myc* oligonucleotide did show some antisense activity towards the growth of HL-60 cells (33%) in comparison to the scrambled control POS sequence (19.5%). If the action of the monothio- modified oligo had been non-specific, the POSC would have shown the same effects as the POS, since each shares the same base composition. That was not the case, however, as the results show. The POS showed greater inhibition of cell proliferation in this experiment than the POSC, thus there must be at least some antisense specific action of the POS anti-*myc* oligo on the HL-60 cells.

The experiments performed most recently give very promising results as pertains to the antisense, sequence specific effects on cell growth by the PS2 modified oligos. All oligos synthesized during the Fall of 1998 and Spring of 1999 were studied: PO2, POS, POS scrambled controls (POSC1, corresponding to the POS sequence and POSC2, corresponding to the PS2 sequence), PS2-1, PS2-2, PS2-3, and PS2 scrambled controls (PS2C-1, PS2C-2, and PS2C-3). PO2 scrambled control (PO2C) was synthesized during previous research and was included in these current experiments. Table 8, Figure 16, Table 9, and Figure 17 demonstrate the results of experiment 1 and Table 10, Figure 18, Table 11, and Figure 19 show the results of the experiment 2 for the Spring of 1999

Table 8. Time and average cell count data for experiment 1, Spring 1999.

					Average Cell Count (x 10^5)								
Time	**Cells**	**Lipofectin**	**PO2**	**PO2C**	**POS**	**POSC-1**	**POSC-2**	**PS2-1**	**PS2-2**	**PS2-3**	**PS2C-1**	**PS2C-2**	**PS2C-3**
0	2.34	2.34	2.34	2.34	2.34	2.34	2.34	2.34	2.34	2.34	2.34	2.34	2.34
24	2.54	2.67	2.55	2.54	1.58	2.27	2.28	1.24	1.01	0.77	1.78	1.92	1.8
48	1.98	2.06	1.79	1.97	1.35	1.73	1.81	0.75	0.74	0.7	1.51	1.39	1.28
96	6.37	5.83	6.82	6.71	5.28	6.42	6.6	4.02	3.74	3.85	6.12	5.68	6.47

Figure 16. Growth curve for experiment 1, Spring 1999, including all PO2, POS, and PS2 oligos, both antisense and scrambled control sequences.

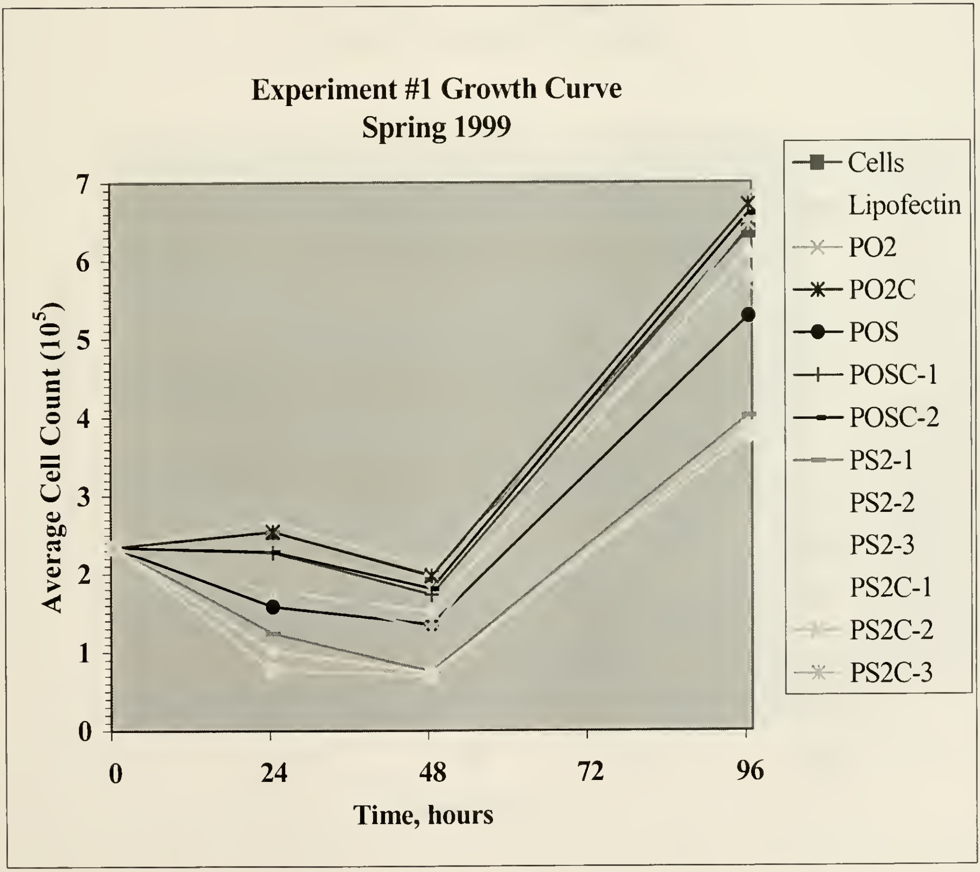
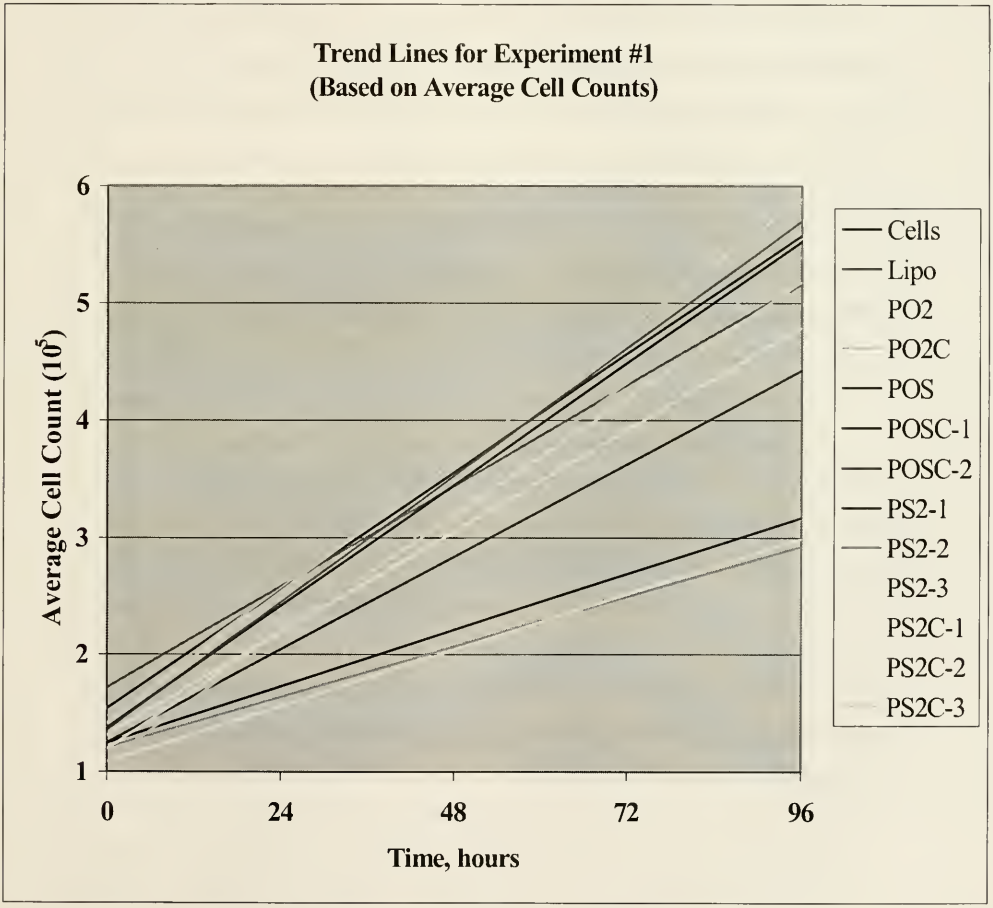


Table 9. Trend data obtained from Microsoft Excel for Experiment #1, Spring 1999. This data gives the best fit line for the average cell counts in Table 8.

			Trend Data for Experiment #1										
Time	**Cells**	**Lipo**	**PO2**	**PO2C**	**POS**	**POSC-1**	**POSC-2**	**PS2-1**	**PS2-2**	**PS2-3**	**PS2C-1**	**PS2C-2**	**PS2C-3**
0	2.34	2.34	2.34	2.34	2.34	2.34	2.34	2.34	2.34	2.34	2.34	2.34	2.34
24	2.54	2.67	2.55	2.54	1.58	2.27	2.28	1.24	1.01	0.77	1.78	1.92	1.8
48	1.98	2.06	1.79	1.97	1.35	1.73	1.81	0.75	0.74	0.7	1.51	1.39	1.28
96	6.37	5.83	6.82	6.71	5.28	6.42	6.60	4.02	3.74	3.85	6.12	5.68	6.47

Figure 17. Trend lines for Experiment #1. This graph can be compared to Figure 16.



Experiment 1 demonstrated all results that were hypothesized in the introduction. The cells alone grew normally throughout the experiment. The cells with Lipofectin™ added verified that the cationic liposome was not toxic, since the cell count was consistent with the cell count of the cells alone throughout the experiment. The PO2 and the PO2C had cell counts comparable to that of the cells alone. If any antisense action had been exhibited by the PO2, it could have occurred in the few hours after oligo addition, but in the times when the cells were counted all PO2 had probably been degraded by cellular nucleases. At the 48 hour cell count, a slight decrease in counts occurred with the cells alone, Lipofectin™ and cells, PO2, PO2C, POSC1, POSC2, PS2C1, PS2C2, and PS2C3. This is most likely attributed to an environmental factor in the controlled incubator, such as depletion of nutrients in the growth medium, as the POS and PS2 antisense oligos also suffered a very slight decreased cell count. The decrease was corrected after the 48 hour point, when fresh medium was added. The POS antisense oligo showed a slight decrease in cell growth (17.1%) as compared to cells alone and the POS scrambled controls (POSC-1 and POSC-2). This decrease was not as signficant as the decreased cell counts encountered with each of the PS2 antisense sequences. PS2-1 showed slightly less of an effect on cell growth (36.9%) when compared to the other two PS2 sequences (41.3% for the PS2-2, 39.6% for the PS2-3). The PS2-1 sequence is modified in the middle of the sequence, thus it is possible that exonucleases could degrade the DNA from the outside of the sequence towards the inside. This is probably not true, since the PS2-1 did cause a significant decrease in cell count when compared to the PO2 and POS sequences. Both the PS2-2 and PS2-3 sequences showed the dramatic decrease in cell growth as the PS2-1 sequence did. This is most notable because the activity of the PS2 sequences is indeed antisense when compared to the effects that the PS2 scrambled controls had on the cells; PS2C-1 had a 3.9% decrease, PS2C-2 had a 10.8% decrease, and PS2C-3 had a 1.6% increase in cell growth compared to cells alone. The three scrambled control PS2 sequences had cell counts consistent with the cells alone. Besides indicating that the sequences are acting in an antisense fashion, the PS2 scrambled controls also verify that the sulfur modification is not toxic to the HL-60 human cells. If there had been any toxicity, the cell counts with the PS2 scrambled controls would have been low compared to the growth of the cells alone.

In the experiment, 4 ml of fresh growth medium supplemented with inactivated serum was added after the 48 hour cell count. The cell density was too dilute to have any reliable cell count at the 72 hour point, so there was no data available for that time period. It was noted to add less medium after 48 hours for the next experiment. A possible explanation for the huge increase in cell growth at 96 hours is that there was fresh medium available for all the cells to grow in. Those tubes of cells with oligo added suffered a large dilution of the oligo concentration in the medium, thus those cells that had not been affected by oligo previously could continue to grow with the new nutrients in the medium. It may be suggested that when new medium is added during the course of an experiment, an amount of oligo should supplement the medium to counter the dilution of the oligo already present.

Table 10. Time and average cell count data for experiment 2, Spring 1999.

					Average Cell Count (10^5)								
Time	**Cells**	**Lipofectin**	**PO2**	**PO2C**	**POS**	**POSC-1**	**POSC-2**	**PS2-1**	**PS2-2**	**PS2-3**	**PS2C-1**	**PS2C-2**	**PS2C-3**
0	1.98	1.98	1.98	1.98	1.98	1.98	1.98	1.98	1.98	1.98	1.98	1.98	1.98
24	2.95	2.59	2.57	2.10	2.25	2.84	2.58	1.57	1.73	1.46	2.38	2.12	2.19
48	2.51	2.51	3.07	2.56	2.05	2.66	3.08	1.66	1.75	1.79	2.60	2.54	2.74
72	5.66	5.02	5.14	5.54	4.63	5.74	5.59	2.88	2.74	2.90	5.74	5.90	5.83
96	7.22	7.06	6.56	7.22	5.28	6.95	6.84	3.61	3.87	3.41	7.24	7.11	7.22

Figure 18. Growth curve for experiment 2, Spring 1999, including all PO2, POS, and PS2 oligos, both antisense and scrambled control sequences.

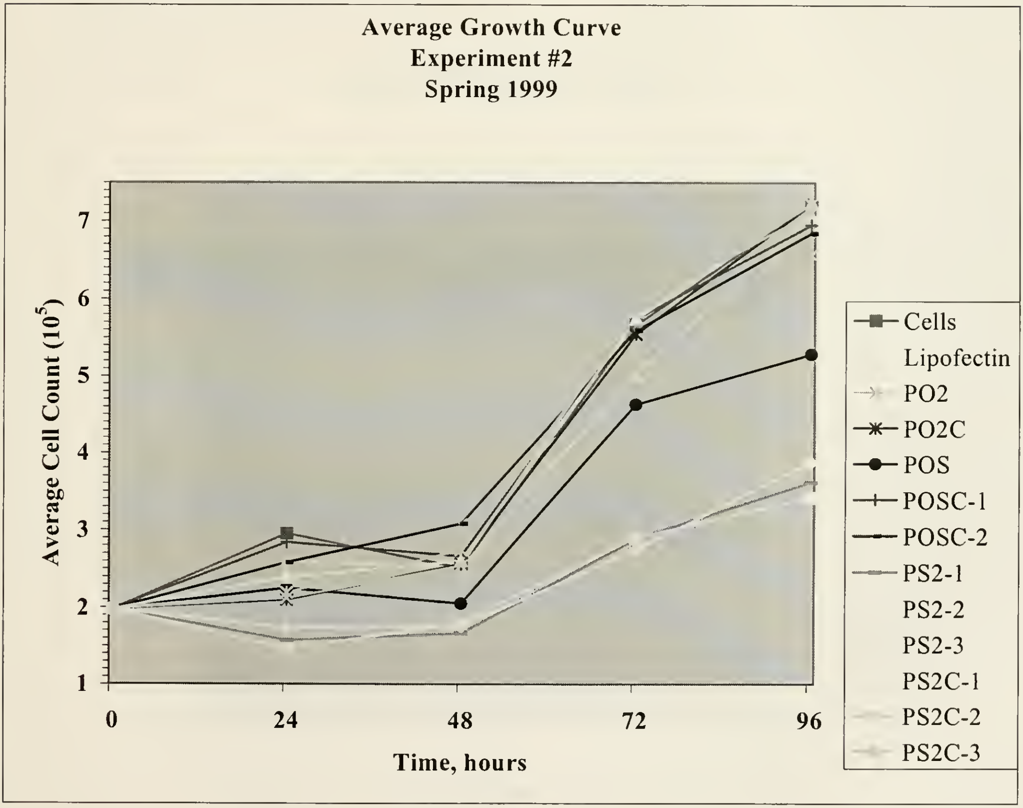


Table 11. Trend data obtained from Microsoft Excel for Experiment #2, Spring 1999. This data gives the best fit line for the average cell counts in Table 10.

				Trend Data for Experiment #2									
Time	**Cells**	**Lipo**	**PO2**	**PO2C**	**POS**	**POSC-1**	**POSC-2**	**PS2-1**	**PS2-2**	**PS2-3**	**PS2C-1**	**PS2C-2**	**PS2C-3**
0	1.43	1.31	1.52	1.10	1.44	1.47	1.47	1.43	1.46	1.45	1.21	1.12	1.17
24	2.75	2.57	2.69	2.49	2.34	2.75	2.74	1.88	1.94	1.88	2.60	2.53	2.58
48	4.06	3.83	3.86	3.88	3.24	4.03	4.01	2.34	2.41	2.31	3.99	3.93	4.00
72	5.38	5.09	5.04	5.27	4.14	5.32	5.29	2.80	2.89	2.74	5.38	5.33	5.40
96	6.70	6.35	6.21	6.66	5.03	6.60	6.56	3.25	3.37	3.17	6.76	6.74	6.82

Figure 19. Trend lines for Experiment #2. This graph can be compared to Figure 18.

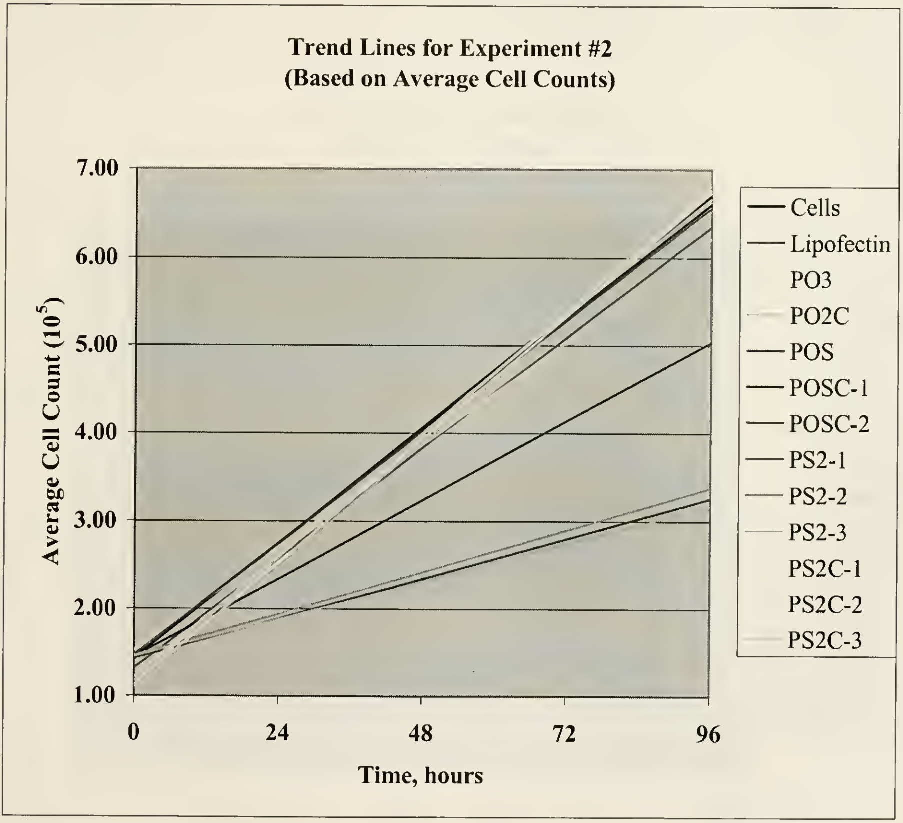
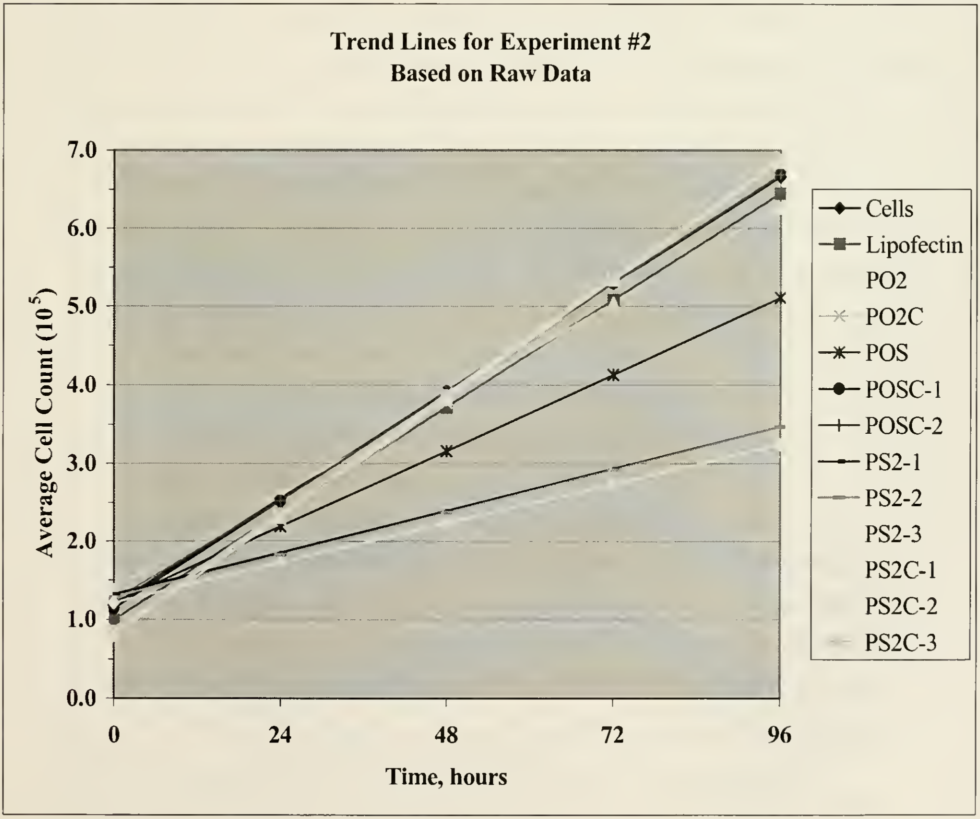


Table 12. Trend data calculated from all raw data cell counts, compared to Table 11 that was calculated based on the average of the cell counts.

					Trend Data based on Raw Data								
Time	**Cells**	**Lipofectin**	**PO2**	**PO2C**	**POS**	**POSC-1**	**POSC-2**	**PS2-1**	**PS2-2**	**PS2-3**	**PS2C-1**	**PS2C-2**	**PS2C-3**
0	1.18	0.99	1.38	0.78	1.21	1.14	1.24	1.32	1.28	1.21	0.85	0.80	0.87
24	2.55	2.36	2.60	2.28	2.19	2.52	2.52	1.85	1.83	1.72	2.35	2.31	2.38
48	3.92	3.72	3.82	3.78	3.16	3.91	3.91	2.39	2.37	2.23	3.86	3.82	3.89
72	5.29	5.08	5.04	5.28	4.13	5.30	5.30	2.93	2.92	2.73	5.36	5.34	5.40
96	6.66	6.45	6.26	6.78	5.11	6.69	6.69	3.47	3.47	3.24	6.87	6.85	6.91

Figure 20. Trend lines for Experiment #2 based on raw data determined best fit lines. This graph was plotted as a means of determining whether results were statistically significant. The lines graphed show approximately the same results as the trend line calculated from the average cell counts. When percent decreases in growth were calculated with the data from Table 12, approximately the same percentages were obtained, within a percentage point. Thus, the results mentioned in the discussion are those calculated based on Table 11.



The purpose of the second experiment was to verify the results of experiment 1 and to get 5 total cell counts for each control and antisense oligo. Only 2 ml of fresh medium supplemented with inactivated serum was added after 48 hours, as opposed to 4 ml as in experiment 1, to allow for sufficient cell density for cell counting at 72 hours. The cells all suffered dilutions of the oligos again, as evidenced by the recovery of cell growth after 48 hours. It is clearly necessary that the fresh medium should be supplemented with oligo. The results of experiment 2 were comparable to experiment 1 in terms of the effects of the oligos on the growth of the HL-60 cells.

In all tubes but those with the POS or the PS2 oligos, cell growth was essentially constant. Once again, as explained in the results for experiment 4 from Spring 1998, any slight variations may be due to experimental error in cell counting. Nonetheless, a significant antisense mechanism of activity is evident for the POS and especially all three PS2 sequences (Figure 18). After the initial cell count at T = 0, the number of cells remained at a lower count, until the addition of fresh medium at the 48 hour point. The increase in cell growth for the POS and PS2 sequences after 48 hours is most likely due to the fact that the concentration of the oligos in the medium was significantly decreased because of the addition of fresh medium. This allowed all cells that had not been affected by the oligos to have more of a chance to grow. However, with the proposed alteration to the experiment, supplementing the fresh medium with an amount of oligo, the difficulties of diluting the oligo would be overcome. This solution may help to determine whether the cells truly are recovering from malnourishment or if the diluted oligo concentration is the factor when fresh medium is added.

Compared to the results of the PS2 oligos from experiment 1, there was not a detectable difference in the effects of the three modifications on the HL-60 cells. In experiment 1, the PS2-1 oligo was slightly less effective at inhibiting cell growth than the PS2-2 or PS2-3 sequence. In experiment 2, all three PS2 oligos had essentially the same cell counts. The PS2-1 sequence decreased cell growth 51.5% compared to the cells alone; PS2-2 had a 49.7% decrease in cell growth; and PS2-3 showed the most dramatic decrease in cell growth at 52.7%. Even the POS antisense oligo demonstrated a significant effect on cell growth in experiment 2, decreasing the cell count by 24.9%.

None of the scrambled control oligos, be it a PO2, POS or a PS2 sequence, had any adverse effect on cell growth. This indicates once more that the results found dictate that the oligos are acting in a sequence specific, or antisense, manner. If the oligos were acting in any other way, the scrambled control oligos should have shown the same effects on cell growth. This experiment also demonstrates that the sulfur modifications are not toxic to the cells. If sulfur modification had been toxic, then the scrambled controls with the same amount and type of sulfur modification would have killed the cells in the same way the antisense sequences did. The experiment proves that this is not the case.

Conclusions

Researchers such as Zamecnik and Stephenson and Holt, Redner, and Nienhuis used unmodified oligos to target oncogenes and were successful in their endeavors (3, 7). The PO2 anti-*myc* sequence used in this research showed no antisense action against c-*myc* in the HL-60 cells, as determined through cell counting. The difference in results may arise from the fact that the experiments were carried out differently, and a decade apart from one another. More importantly, the experiments were carried out with different cell types and different assay procedures. Holt, Redner, and Nienhuis incubated the unmodified oligos in the growth medium with the HL-60 cells in 1988 and allowed for random uptake by the cells. They recognized the half-life of the unmodified oligos, but still obtained decreased c-*myc* protein expression. Their studies focused on the first few hours after incubation for detecting oligo action (7).

In 1998-99, this research used modified antisense oligos in addition to the unmodified oligos to study effects on HL-60 cells. Lipofectin™ was used as a means of introducing the antisense oligos into the cells, rather than just allowing for random uptake of the oligos. Thus, the cellular concentrations of antisense oligos probably differed between these experiments and those of Holt, Redner, and Nienhuis. Two factors may contribute to the differences in the experimental results. The experiments described here lasted for 96 to 120 hours, with measurements of cell growth taken every 24 hours by means of cell counting. Holt, Redner, and Nienhuis studied the cells just 4 hours after transfection and incubation and determined oligo action by measuring protein expression.

This research focused on the long-term stability of the antisense oligonucleotides in the inhibition of HL-60 cell growth.

In investigating the effects of anti-*myc* on cell growth in this research, cell counting was the only means of determining whether cells grew or not. It is possible that other assays could be used to study HL-60 proliferation. Holt, Redner, and Nienhuis suggest that HL-60 cells are induced to differentiate when c-*myc* expression is decreased. A test for differentiation, such as expression of a protein or antibody, could be developed to study the effects of anti-*myc* oligos. These researchers also note that differentiated HL-60 cells do not grow rapidly like the HL-60 cells that overexpress c-*myc* (7); this could be the cause of the decrease in cell counts after addition of the antisense oligos. Soft agar assays are possible with HL-60 cells because proliferating HL-60 cells will grow in semi-solid media (5). If c-*myc* expression is decreased by anti-*myc* oligo action, the cells will not be able to grow in the soft agar. This assay will not give the quantitative results of cell counting; it also takes a long time to achieve results because the cells have to grow several days in the agar.

Further research with modified antisense oligonucleotides was imperative after the Spring of 1998 studies that were performed with only PO2 and POS oligos. Studies during the Fall of 1998 and Spring of 1999 were done with the dithiophosphate oligos to determine their effects on HL-60 growth in comparison to the demonstrated effects of PO2 and POS anti-*myc* oligos.

The POS oligo was shown to demonstrate some type of antisense activity in the preliminary experiments, and once PS2 oligos and their scrambled control sequences were synthesized and purified, conclusive experiments could be carried out. Indeed, the PS2 oligos showed an inhibition of HL-60 cell growth as hypothesized, decreasing the growth of cells up to as much as 53%. Each modification of the PS2 oligo (middle, end, and middle and end together) affected cell growth in approximately the same way; there may have been some nuclease degradation of the PS2-1 sequence, but its effects on cell growth did not differ much from that of the PS2-2 and PS2-3.

These experimental results seem to support the antisense theory model. If an antisense oligonucleotide does indeed act in a sequence specific, antisense manner to target a gene that is overexpressed in cancer or leukemia cells, gene expression can be

inhibited and cell growth can be terminated. The application of this theory offers the potential to halt the growth of cancer cells if the overexpressed gene can be targeted *in vivo*. The hopes for the future include studying other cell types for similar effects by targeting different overexpressed genes using different antisense oligonucleotides.

The difficulties in this research arise not in the cell culture aspect but in the synthesis and purification procedures for the oligonucleotides. The yields of the oligos are significantly low, all below 10% of the theoretical 0.2 μmol synthesis yield. When an oligo is removed from the cartridge it was synthesized on, the theoretical yield is 0.2 μmol. There are several steps that the oligo undergoes before it is finally prepared for addition to cell cultures. This gives much opportunity for loss of the oligo, whether due to small amounts remaining in a tube or in a pipette tip, or due to experimental error during the procedures. Lyophilization of the oligo may be where much of an oligo is lost. After lyophilization is complete, the oligo is resuspended in a very small amount of ultrapure water. The oligo is dried to the sides of a siliconized flask, so it is difficult to know when all the oligo has been dissolved. It is highly likely that much of the freeze-dried oligo remains in the flask. In order to determine where in the procedures the oligo is lost the most, a study of UV/Vis spectrophotometry measurements after each step is necessary. This may help to improve the final yields if a certain step is targeted as where the product is lost. Higher yields of the antisense oligonucleotides would contribute to a more feasible application in *in vivo* studies.

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